

UNITED STATES BANKRUPTCY COURT
EASTERN DISTRICT OF NEW YORK

-----X
In re

EUROSPARK INDUSTRIES, INC.
Debtor.

Chapter 11

Case No. 98-21459-CEC
-----X

DECISION

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CARLA E. CRAIG
Chief United States Bankruptcy Judge

This matter comes before the Court on the motion of the United States Trustee (the “UST”) for the appointment of a chapter 11 trustee in this case pursuant to 11 U.S.C. § 1104(a)(2) and (a)(3).¹ Eurospark Industries, Inc. (“Eurospark” or the “Debtor”) and its sole shareholder, Michael Spiegel, object to the appointment of a chapter 11 trustee. The UST’s motion is supported by the estate’s largest secured creditor, Fleet Precious Metals, Inc., (“Fleet”);² the Debtor’s former attorneys, Windels Marx Lane & Mittendorf, LLP; the Debtor’s current bankruptcy counsel, Kane Kessler, P.C.; and the Debtor’s current special counsel, Weg & Myers, P.C. (“Weg & Myers”).³ For the following reasons, the appointment of a trustee is warranted pursuant to § 1104(a)(2).

Jurisdiction

This Court has jurisdiction of this core proceeding pursuant to 28 U.S.C. §§ 157(b)(2)(A) and 1334, and the Eastern District of New York standing order of reference dated August 28, 1986. This decision constitutes the Court’s findings of fact and conclusion of law to the extent required by Federal Rule of Bankruptcy Procedure 7052.

Background

The following facts are undisputed.

1. The Bankruptcy Filing

On August 14, 1998, Eurospark filed a voluntary petition under chapter 11 of the Bankruptcy Code. The Debtor was in the business of fabricating fine gold into jewelry.

¹ Unless otherwise specified, all statutory references herein are to the Bankruptcy Code, 11 U.S.C.

² Fleet appears by its successor in interest, Bank of America.

³ On March 5, 2009, the Debtor filed applications to substitute Kane Kessler and Weg & Myers with Gabor & Marotta, LLC. (Docket entry nos. 192, 194). These applications are still pending.

(Proposed Disclosure Statement at 13, docket entry # 110.) Prior to filing, on March 14, 1998, the Debtor's gold, valued by the Debtor in excess of \$4 million, was allegedly stolen during an armed robbery. (Proposed Disclosure Statement at 18, docket entry #110.)

During the first year of the bankruptcy case, the Debtor, with court approval, sold all of its machinery, equipment, inventory and real property. On July 7, 1999, the Debtor filed a proposed plan of liquidation and disclosure statement, to which Fleet objected. Thereafter, on September 23, 1999, the Court approved a stipulation between the Debtor and Fleet resolving Fleet's objections, and pursuant to which the parties agreed to submit a consensual plan of liquidation and disclosure statement. However, more than a decade later, no other plan or disclosure statement, consensual or otherwise, has ever been filed.

2. The Adversary Proceedings

In September 1998, the Debtor commenced two adversary proceedings (the "Adversary Proceedings") against Massachusetts Bay Insurance Company ("Massachusetts Bay"), the Underwriters at Lloyd's, and Lloyd Thompson Limited Art Incorporated N.V. (together, "Lloyd's," and with Massachusetts Bay, the "Insurance Companies"). The Adversary Proceedings sought to recover proceeds of two insurance policies based upon the Debtor's allegation that the Debtor lost gold valued in excess of \$4 million and business income in excess of \$1.5 million as a result of the armed robbery. The maximum coverage under the insurance policies is \$6.5 million.⁴ Among other affirmative defenses, the Insurance Companies asserted

⁴ The Debtor contends that, if a favorable verdict is rendered in the Adversary Proceedings, the estate could recover, with interest, approximately \$8 million.

that Mr. Spiegel staged the alleged robbery, that the policies are void, and that the policies do not cover the alleged losses.

On July 20, 1999, the Debtor filed motions in the Adversary Proceedings to withdraw the reference to the District Court. On December 2, 1999, the District Court denied the motion without prejudice to renewal after completion of discovery. Subsequently, on January 13, 2005, after discovery was completed, the District Court issued an order withdrawing the reference.

On May 5, 2005, Mr. Spiegel, acting on his own behalf, filed a motion to intervene in the Adversary Proceedings. In support of the motion, Mr. Spiegel asserted that his individual interests are not aligned with those of the Debtor. Mr. Spiegel stated:

The interest of the debtor corporation, Eurospark Industries, Inc. is to recover an award in bankruptcy in order to pay the claims of Eurospark's various creditors and its legal counsel. Spiegel's interests, however, are to recover Spiegel's individual money, time and labor lost as a result of [the Insurance Companies'] wrongful actions. Clearly the interests of the corporation and of the individual are divergent. Spiegel's interest cannot be represented by that of the [D]ebtor, as his interests conflict with those of the [D]ebtor.

(UST Ex. 1 at 5; Reply Mem. of Law at 5, No. 1:05-cv-00208-ENV-JMA, docket entry #13.)

On June 9, 2005, the District Court granted Mr. Spiegel's motion to intervene in the Adversary Proceedings.

On September 28, 2005, and on December 30, 2005, Massachusetts Bay and Lloyd's filed motions for summary judgment. The Debtor and Mr. Spiegel opposed the motions.

On February 1, 2008, Magistrate Judge Joan M. Azrack issued a Report and Recommendation denying Massachusetts Bay's motion, but granting Lloyd's motion to the extent it related to the Debtor's claim for indemnification for the labor component of the gold.

Thereafter, on July 2, 2008, The Honorable Eric Vitaliano issued a Memorandum and Order adopting the Report and Recommendation without modification.

After the District Court ruled on the summary judgment motions, the Debtor, by special counsel Weg & Myers, engaged in mediation with the Insurance Companies. As a result of the mediation, a proposed settlement resolving the Adversary Proceedings was reached, under which the Insurance Companies would pay \$2,225,000, from which Fleet would receive \$2 million in satisfaction of its secured claim, and the Debtor's administrative creditors would share \$225,000. Currently, Eurospark's debt consists of \$2.7 million secured debt owed to Fleet, approximately \$1 million of administrative claims,⁵ approximately \$250,000 of unsecured claims, and a \$1.2 million subordinated claim held by Mr. Spiegel. (Tr.⁶ 6/4/09 at 9-11, 21; Tr. 8/12/09 at 28; Proposed Disclosure Statement at 33, docket entry #110.) The unsecured creditors will receive no distribution under the terms of the proposed settlement, nor will Mr. Spiegel. Fleet and the administrative claimants support the proposed settlement, and Mr. Spiegel objects to it.

The Debtor, by Mr. Spiegel, and acting through proposed substitute counsel Gabor & Marotta, LLC, argues that no settlement was reached because Mr. Spiegel, the principal of the Debtor and its sole officer, did not consent to it. Mr. Spiegel believes that this settlement is inadequate, and that the claim against the Insurance Companies should be litigated to a final determination.

⁵ This figure does not include a \$500,000 contingency fee for Weg & Myers, which will be paid by Fleet pursuant to a stipulation dated April 23, 2001.

⁶ "Tr." refers to the transcript of the hearing held on the date specified.

To resolve this impasse, the UST seeks the appointment of a chapter 11 trustee for the estate pursuant to § 1104(a) of the Bankruptcy Code. Given that the only remaining assets of the estate are the claims against the Insurance Companies, the trustee's principal role would be to evaluate the proposed settlement and to determine whether to seek approval of it, or to administer these assets in some other way, such as by proceeding with litigation or pursuing further settlement discussions.

Legal Standard

As applicable to this case, § 1104(a) of the Bankruptcy Code provides:⁷

At any time after the commencement of the case but before confirmation of a plan, on request of a party in interest or the United States trustee, and after notice and a hearing, the court shall order the appointment of a trustee -

- (1) for cause, including fraud, dishonesty, incompetence, or gross mismanagement of the affairs of the debtor by current management, either before or after the commencement of the case, or similar cause, but not including the number of holders of securities of the debtor or the amount of assets or liabilities of the debtor; or
- (2) if such appointment is in the interests of creditors, any equity security holders, and other interests of the estate, without regard to the number of holders of securities of the debtor or the amount of assets or liabilities of the debtor.

11 U.S.C. §1104(a).

The Bankruptcy Code generally permits chapter 11 debtors to remain in control of their assets and business operations. In re Adelphia Commc'ns. Corp., 336 B.R. 610, 655 (Bankr.

⁷ Section 1104(a)(3) was added to the Bankruptcy Code by the Bankruptcy Abuse Prevention and Consumer Protection Act of 2005. Because this case was filed in 1998, the 2005 amendments do not apply. In re Adelphia Commc'ns. Corp., 336 B.R. 610, 655 n.97 (Bankr. S.D.N.Y. 2006). Accordingly, the UST's motion must be denied to the extent it seeks to appoint a chapter 11 trustee pursuant to § 1104(a)(3).

S.D.N.Y. 2006). A debtor-in-possession owes fiduciary duties to the bankruptcy estate. Smart World Techs., LLC v. Juno Online Servs., Inc. (In re Smart World Techs., LLC), 423 F.3d 166, 175 (2d Cir. 2005) (stating that a debtor-in-possession's "duty to wisely manage the estate's legal claims is implicit in the debtor's role as the estate's only fiduciary"); In re Bowman, 181 B.R. 836, 843 (Bankr. D. Md. 1995). These obligations "include a duty of care to protect the assets, a duty of loyalty and a duty of impartiality." Bowman, 181 B.R. at 843 (citing Daniel B. Bogart, Liability of Directors of Chapter 11 Debtors in Possession: "Don't Look Back-Something May Be Gaining on You", 68 Am. Bankr. L.J. 155, 216-27 (1994)). To fulfill its duty of loyalty, a debtor-in-possession must "avoid self-dealing, conflicts of interest and the appearance of impropriety." Id.

"When a debtor-in-possession is incapable of performing [its statutory] duties, a Chapter 11 trustee may be appointed." In re Ionosphere Clubs, Inc., 113 B.R. 164, 169 (Bankr. S.D.N.Y. 1990). Nonetheless, the "appointment of a trustee should be the exception, rather than the rule,"" Adelphia, 336 B.R. at 655 (quoting In re Sharon Steel Corp., 871 F.2d 1217, 1225 (3d Cir. 1989)), and is an extraordinary remedy, In re Ridgemour Meyer Props., LLC, 413 B.R. 101, 108 (Bankr. S.D.N.Y. 2008); In re Euro-Am. Lodging Corp., 365 B.R. 421, 426 (Bankr. S.D.N.Y. 2007); Adelphia, 336 B.R. at 655. The movant must prove, by clear and convincing evidence, that the appointment of a chapter 11 trustee is warranted. Ridgemour Meyer, 413 B.R. at 108; Euro-Am. Lodging, 365 B.R. at 426; Adelphia, 336 B.R. at 655.

It is not necessary to find fault on the part of the debtor before appointing a chapter 11 trustee in "the interests of creditors, any equity security holders, and other interests of the estate" pursuant to § 1104(a)(2). Sharon Steel, 871 F.2d at 1226; Euro-Am. Lodging, 365 B.R. at 428;

In re 1031 Tax Group, LLC, 374 B.R. 78, 90 (Bankr. S.D.N.Y. 2007). When deciding whether relief under § 1104(a)(2) is warranted, a court will consider:

- (i) the trustworthiness of the debtor;
- (ii) the debtor in possession's past and present performance and prospects for the debtor's rehabilitation;
- (iii) the confidence-or lack thereof-of the business community and of creditors in present management; and
- (iv) the benefits derived by the appointment of a trustee, balanced against the cost of the appointment.

Ionosphere, 113 B.R. at 168 (citations omitted). Notwithstanding the articulation of these factors, the standard under § 1104(a)(2) is flexible. Ridgemour Meyer, 413 B.R. at 112; Euro Am. Lodging, 365 B.R. at 427; Adelphia, 336 B.R. at 658, Ionosphere, 113 B.R. at 168.

Ultimately, the court should consider the “practical realities and necessities” of the case.

Ionosphere, 113 B.R. at 168 (quoting In re Hotel Assocs., Inc., 3 B.R. 343, 345 (Bankr. E.D. Pa. 1980)).

Discussion

The UST argues that the appointment of a trustee pursuant to § 1104(a)(2) is appropriate, because in the present posture of the case, where there are no ongoing business operations, no assets other than the insurance claims, and no reorganization in prospect, Mr. Spiegel's interest in obtaining a recovery for himself individually constitutes a conflict of interest that prevents him from exercising the debtor's fiduciary duty to administer the claims against the Insurance Companies for the benefit of creditors of the estate. The UST points out that Mr. Spiegel admitted – indeed, asserted – in his motion to intervene in the Adversary Proceedings, that his

interests with regard to the claims against the Insurance Companies “conflict with those of the Debtor.” (UST Ex. 1 at 5; Reply Mem. of Law at 8, No. 1:05-cv-00208-ENV-JMA, docket entry #13.) While the UST does not question Mr. Spiegel’s honesty, she argues that a chapter 11 trustee is needed to independently evaluate the proposed settlement.

At the outset, the Debtor argues that this Court does not have jurisdiction to hear the UST’s motion because the District Court withdrew the reference of this case. This argument is contradicted by the terms of the District Court order. A district court may “withdraw, in whole or in part, any case or proceeding referred” to the bankruptcy court. 28 U.S.C. § 157(d). Here, the District Court withdrew only the reference of the Adversary Proceedings:

[I]t is SO ORDERED that the adversary proceedings bearing number 198-1499-260 and 198-1514-260, originally referenced to this Court on July 26, 1999, are now accepted by this Court and these proceedings . . . will now be transferred back to this Court’s docket for all purposes through their resolution

(Order dated January 13, 2005 at 3, No. 1:05-cv-00208-ENV-JMA, docket entry #1.)

The reference of the bankruptcy case was not withdrawn; accordingly, this Court has jurisdiction to hear any motions made in the bankruptcy case, including the UST’s motion to appoint a chapter 11 trustee.

The Debtor argues that Mr. Spiegel was excluded from the mediation sessions with the Insurance Companies, and that he has the sole authority to accept a settlement on behalf of the Debtor. Because Mr. Spiegel did not accept the settlement offer on the Debtor’s behalf, the Debtor argues that appointment of a trustee is premature, as there is no settlement for a chapter 11 trustee to evaluate. The Debtor further argues that the disclosure of the proposed settlement

violates Rule 408 of the Federal Rules of Evidence, and cannot be considered in connection with the UST's motion.

These arguments are meritless. Whether or not the Debtor accepted the settlement, at a minimum, there is a settlement offer by the Insurance Companies that a chapter 11 trustee could consider. Rule 408 of the Federal Rules of Evidence does not prevent this Court from considering that fact, or the proposed settlement's terms. Rule 408 only prohibits disclosure of "conduct or statements made in compromise negotiations regarding the claim" for the purposes of establishing "liability for, invalidity of, or amount of a claim that was disputed as to validity or amount, or to impeach through a prior inconsistent statement or contradiction." Fed. R. Evid. 408(a). The settlement offer was not disclosed to establish the validity or amount of the Debtor's claims against the Insurance Companies, or for any other purpose that would be prohibited by Rule 408; rather, it was disclosed to set forth the factual background for the UST's motion.

The UST argues that appointment of a chapter 11 trustee is warranted under § 1104(a)(2) because Mr. Spiegel's interests are not aligned with the estate's interests. In support of this argument, the UST introduced into evidence Mr. Spiegel's memorandum of law in support of his motion to intervene in the Adversary Proceedings, where he stated that his interests "are divergent" from the Debtor's. Mr. Spiegel asserted that he should be permitted to intervene because his interests conflict with the estate's interests, because while the Debtor seeks to recover an award to pay creditors, Mr. Spiegel seeks to "recover [his] individual money, time and labor lost." (UST Ex. 1, Spiegel Reply Mem. of Law at 5, No. 1:05-cv-00208, docket entry #13.) Evidently persuaded by Mr. Spiegel's argument, the District Court granted Mr. Spiegel's motion to intervene.

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TGF- β -induced RhoA and p160^{ROCK} activation is involved in the inhibition of cdc25A with resultant cell cycle arrest

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Running Title: TGF- β -mediated p160^{ROCK} inhibition of cdc25A

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The ability of the TGF- β signaling pathways to inhibit proliferation of many epithelial and hematopoietic cells while stimulating proliferation of fibroblasts remains a conundrum. Here we report that absence of RhoA and p160^{ROCK} activity in fibroblastic NIH3T3 cells and its presence in epithelial NMuMG cells can at least partially explain the difference in the TGF- β growth response. Further, evidence is presented for TGF- β stimulated p160^{ROCK} translocation to the nucleus and phosphorylation and inhibition of the cdk-activating phosphatase, cdc25A, with resultant inhibition of cdk2 activity and G1-S progression in NMuMG cells. These results provide novel evidence that signaling through RhoA and p160^{ROCK} is important in TGF- β inhibition of cell proliferation and links signaling components for epithelial transdifferentiation with regulation of cell cycle progression.

Transforming growth factor- β 1 (TGF- β 1) causes growth inhibition in many non-transformed epithelial cell types (Akhurst and Deryck, 2001). The mechanism by which this occurs involves the binding and activation of TGF- β type I and type II receptors, for the subsequent induction of parallel downstream signaling pathways (Massague et al., 2000). The established paradigm of TGF- β type I receptor phosphorylation of Smad2 and Smad3 in conjunction with Smad4 recruitment is important in TGF- β -mediated G1 cell cycle arrest (Kretzschmar and Massague, 1998). The SMAD signaling pathway up-regulates the expression of cyclin-dependent kinase (cdk) inhibitors, p16^{INK4}, p15^{INK4}, p27^{Kip1}, and p21^{Cip1}, thereby suppressing cyclin D and/or cyclin E associated hyper-phosphorylation of Rb and S phase progression (reviewed in (Massague et al., 2000)). Additionally, SMAD signaling is associated with the transcriptional down-regulation of the growth stimulatory factor, c-myc (Alexandrow and Moses, 1995; Feng et al., 2002; Yagi et al., 2002). This in turn down-regulates the cdk activating phosphatase, cdc25A (Jinno et al., 1994), a transcriptional target of c-myc (Bernardi et al., 2000; Galaktionov et al., 1996). However, fibroblastic NIH3T3 cells exhibit both TGF- β -mediated SMAD activation and *c-myc* down-regulation, yet proliferation of these cells is stimulated by TGF- β (Koskinen et al., 1991). Earlier findings suggest that growth stimulation of NIH3T3 cells by TGF- β is due to increased cyclin E associated kinase activity (Sgambato et al., 1997).

We previously described TGF- β activation of RhoA in various cell types with the exception of NIH3T3 cells (Bhowmick et al., 2001a). Thus, we hypothesized that RhoA signaling may be involved in TGF- β -mediated cell cycle arrest. The role of RhoA in cytoskeletal organization is well established, and it has also emerged as a mediator of cell cycle progression (Kimura et al., 2000; Olson et al., 1998; Song et al., 2000). The activation-state of RhoA is positively regulated by guanine exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (Hall, 1998). Downstream effector proteins of RhoA, such as p160^{ROCK}, PKN, mDia, rotekin, and citron, have been reported to mediate many specific processes (Aspenstrom, 1999). The role of p160^{ROCK} and mDia are better understood as mediators of the formation and maintenance of stress fibers. Recently, PKN activation has been implicated in the delay of G2-M progression (Misaki et al., 2001). However, since physiological activation of RhoA is primarily a component of cytokine signaling, where other cytokine-specific signals are present, RhoA activity occurs in both a stimulus- and context-dependent manner.

Here we define the role of RhoA in the context of TGF- β regulation of the G1 cell cycle arrest. Our results suggest that TGF- β -mediated p160^{ROCK} activation is involved not only in epithelial to mesenchymal transdifferentiation (EMT) as we demonstrated previously (Bhowmick et al., 2001a), but also the phosphorylation of cdc25A and inhibition of cdk2 activity. This suggests a common pathway by which cells regulate growth inhibition and the actin cytoskeleton organization.

Results

TGF- β -mediated growth arrest involves RhoA activation. Treatment of NMuMG cells, with TGF- β results in growth inhibition, however NIH3T3 cells are growth stimulated when treated with TGF- β (Sgambato et al., 1997). To identify TGF- β signaling pathways involved in growth stimulation of NIH3T3 cells, we initially analyzed SMAD signaling in both cell types. We found the SMAD pathway to be active, as illustrated by 3TP-Lux (containing PAI-1 and collagenase promoter elements) and CAGA (SMAD binding consensus site) reporter activation (Figure 1A). Both cell lines showed an 10-fold 3TP-Lux and an 3-fold induction of CAGA reporter activity. As a transcriptional reporter for RhoA activity we also examined induction in serum response element (SRE) by TGF- β and found that NMuMG cells showed a 3-fold induction while NIH3T3 cells had little TGF- β -mediated activation. The transcriptional reporter experiments were supported by western blotting for the phosphorylation of Smad2, indicating Smad2 activation by 1 h of TGF- β treatment of both NMuMG and NIH3T3 cells (Figure 1B). The expression levels of Smad2 did not change in either cell line for the 12h of TGF- β treatment. However, TGF- β stimulated GTP-RhoA accumulation in NMuMG cells by 5 min, but did

not induce RhoA activation in NIH3T3 cells, as has been previously shown (Figure 1C) (Bhowmick et al., 2001a). The total RhoA expression levels did not change, and lysophosphatidic acid (LPA) stimulated RhoA activation in both NMuMG and NIH3T3 cells.

We next examined the disparity in TGF- β signaling and growth inhibition in NIH3T3 cells by artificially stimulating the RhoA signaling pathway. Initially, 3 H-thymidine incorporation of NIH3T3 cells retrovirally transduced with wt-RhoA was compared to that of NIH3T3 cells infected with empty virus. In the presence of increasing concentrations of TGF- β , there was a 25% increase in 3 H-thymidine incorporation of NIH3T3 cells, while wt-RhoA over-expressing NIH3T3 cells were no longer growth stimulated (Figure 2A). We hypothesized that a more physiological method of RhoA activation might be required to mimic the transient RhoA activation by TGF- β in NMuMG cells. Thus a RhoA-GEF, Ost, was retrovirally transduced into NIH3T3 cells. Sixty percent growth inhibition was achieved in Ost expressing NIH3T3 cells in the presence of 10 ng/ml TGF- β . To further examine the role of RhoA activation in the presence and absence of TGF- β , we performed flow cytometry on rapidly growing NIH3T3 and Ost expressing NIH3T3 cell. Treatment with TGF- β or expression of Ost alone did not significantly alter the cell cycle profile compared to control cells (Figure 2B). However, in Ost-expressing NIH3T3 cells TGF- β elevated the G1 fraction of cells by 2 fold. In contrast, TGF- β increased the fraction of Ost-expressing NIH3T3 cells in the G1 phase of the cell cycle two-fold indicating a G1 arrest. The retroviral infection of GFP cDNA showed greater than 95% infection efficiency in the NMuMG and NIH3T3 cells ((Bhowmick et al., 2001a) and data not shown),

Conversely, when RhoA signaling was specifically inhibited by the stable introduction of the RhoA-binding domain of rhotekin (RBD) (Ren et al., 1999; Welsh et al., 2001) in NMuMG cells, TGF- β -mediated growth inhibition was abrogated (Fig 3A). The results of these studies suggest a role for RhoA activation in TGF- β -mediated cell cycle arrest.

P160^{ROCK} contributes to TGF- β -mediated growth inhibition. We examined two downstream effectors of RhoA signaling, PKN and p160^{ROCK}, for their possible involvement in TGF- β -mediated growth inhibition. Dominant-negative PKN and p160^{ROCK} constructs were retrovirally transduced into NMuMG cells. Dominant-negative PKN-transduced NMuMG cells displayed a similar dose response of TGF- β -mediated growth inhibition as control vector infected cells (Figure 3B). In addition, treatment with a PKN inhibitor, 6-thioguanine, gave similar results (data not shown). However, TGF- β treatment of dominant-negative p160^{ROCK} (Ishizaki et al., 1997) expressing cells displayed no growth inhibition. There was little effect on 3 H-thymidine incorporation by the expression of dominant-negative p160^{ROCK} alone, as reported by others similar results were found by the treatment of epithelial cell lines, MK, Mv1Lu, and NMuMG cells with a p160^{ROCK} specific inhibitor, Y27632 (Sahai et al., 1999; Uehata et al., 1997) (Figure 3C). Further, treatment with increasing concentrations of Y27632 in the presence of 1 ng/ml TGF- β (a concentration sufficient to mediate $\geq 50\%$ growth inhibition alone) showed antagonism of growth inhibition. Similarly, when TGF- β concentrations were varied in the presence of 5 μ M Y27632, there was reduced growth inhibitory response (data not shown).

To address whether Y27632-associated blocking of TGF- β -mediated inhibition of DNA synthesis, as measured by 3 H-thymidine incorporation, correlated with effects on cell proliferation cell counting experiments were performed for a period of 3 days of treatment of NMuMG cells. While TGF- β treatment resulted in reduced cell numbers, the combination treatment of Y27632 and TGF- β displayed similar rates of proliferation with that of untreated cells grown in serum containing medium through the times course examined (Figure 3D). Together these results suggest a role for p160^{ROCK} activation in TGF- β -mediated growth arrest.

TGF- β -mediated phosphorylation of cdc25A involves p160^{ROCK} activity. Next we examined the impact of p160^{ROCK} signaling on TGF- β -mediated regulation of select proteins involved in cell cycle

regulation at the G1/S boundary. This was achieved by examining rapidly growing NMuMG cells treated with 5 ng/ml TGF- β for 0, 24, or 48 h either in the presence or absence of 5 μ M Y27632. Cell lysates were examined for changes in Rb, p160^{ROCK}, cdc25A, and α -tubulin expression and phosphorylation by Western blotting. In the absence of TGF- β , hyperphosphorylated Rb was present and there was high basal cdk2 activity, and cdc25A expression (Figure 4). TGF- β treatment resulted in the appearance of predominantly hypo-phosphorylated Rb by 24 h while the co-treatment with Y27632 delayed this process by 24 h. TGF- β did not alter the expression of p160^{ROCK} but 48h treatment resulted in significant down regulation of cdc25A expression. Y27632 had little effect on the expression of p160^{ROCK} or cdc25A. The expression level of α -tubulin was constant and used as a control for protein loading. To determine whether the delay in Rb hypophosphorylation resulting from the co-treatment of TGF- β and Y27632 was due to altered cdk2 activity, *in vitro* kinases for immuno-precipitated cdk2 were performed. Y27632 antagonized TGF- β -inhibition of cdk2 kinase activity. Thus, these studies suggest an important role for p160^{ROCK} in TGF- β -mediated regulation the state of Rb phosphorylation and cdk2 activity.

Since cdc25A is an important regulator of cdk2 activity, we chose to test whether TGF- β can regulate cdc25A activity in a p160^{ROCK}-dependent manner by analyzing TGF- β effects on the phosphorylation of cdc25A utilizing both *in vivo* and *in vitro* kinase assays. *In vivo* kinase assays were performed by metabolic labeling of NMuMG cells with [³²P]-orthophosphate followed by immuno-precipitation of cdc25A. This procedure showed low basal level of cdc25A phosphorylation in rapidly growing cells absent of exogenous TGF- β (Figure 5A, lane 1). A successive increase in cdc25A phosphorylation was detected in cells 30 min, 1h, and 3 h following TGF- β addition (Figure 5A, lane 2-4). The concomitant addition of Y23637 inhibited TGF- β -stimulated cdc25A phosphorylation (Figure 5A, lane 5-6). However, since we were not able to co-precipitate cdc25A and p160^{ROCK} (data not shown), it is possible that p160^{ROCK} regulates cdc25A phosphorylation, but is not the kinase that directly acts on cdc25A. Thus, the regulation of cdc25A phosphorylation was directly determined by immuno-precipitating p160^{ROCK} and cdc25A from NMuMG and NIH3T3 cells for use in *in vitro* kinase assays. P160^{ROCK}-associated cdc25A phosphorylation activity reached maximal levels by 1h of TGF- β treatment and maintained an elevated level by 3h of treatment in NMuMG cells (Figure 5B). In contrast, TGF- β did not stimulate p160^{ROCK} activity in NIH3T3 cells, consistent with the lack of TGF- β -mediated RhoA activity in these cells.

Further evidence for a role of RhoA signaling in cdc25A phosphorylation was determined by expressing Ost in NIH3T3 cells and examining TGF- β -stimulation of cdc25A phosphorylation *in vivo*. [³²P]-orthophosphate labeled Ost-expressing NIH3T3 cells immuno-precipitated for cdc25A after 0, 1, and 3h of TGF- β treatment showed increased phosphate labeling, whereas no labeling was observed in the absence of Ost expression (Figure 5C). This is in agreement with results from the *in vitro* kinase assay (Figure 5B). Together, these results indicate that TGF- β activates cdc25A phosphorylation *in vivo* through a RhoA- and p160^{ROCK}-dependent manner.

TGF- β inhibits cdc25A phosphatase activity. To better understand the mechanism of cdc25A phosphorylation, cell fractionation experiments were performed examining TGF- β -mediated p160^{ROCK} sub-cellular localization. Nuclear and cytoplasmic fractions were separated from NMuMG cells treated with TGF- β over a time course of 12h. As a control, TGF- β stimulation of Smad2 was examined, showing elevated detection in the nuclear fraction by 1h of treatment and persisting through 12h of treatment (Figure 6A). Nuclear translocation of p160^{ROCK} was apparent at the 1h and 3h time points, while changes in the cytoplasmic expression of p160^{ROCK} were not detectable within the 12 hours of TGF- β incubation. Cdc25A was only detected in the nuclear fraction. The cell fractions were also examined for PCNA and RhoGDI expression as a control for nuclear and cytoplasmic

fractionations, respectively. There was no perceptible cytoplasmic expression of PCNA, and there was minimal RhoGDI detected in the nuclear fraction.

The impact of TGF- β -mediated cdc25A phosphorylation on its enzymatic activity was determined by *in vitro* phosphatase assays. NMuMG cells were harvested after treatment with TGF- β (5 ng/ml) through a 6 h time course in the presence and absence of Y23637 (5 μ M). We found that the incubation of the phosphorylated-histone H1 substrate with immuno-precipitated cdc25A from rapidly growing cells showed efficient phosphatase activity. In contrast, cdc25A from TGF- β -treated cells had diminished phosphatase activity as early as 30 min with progressively greater inhibition up to 6 h (Figure 6B). Treatment with Y23637 antagonized the TGF- β inhibition of cdc25A de-phosphorylation of histone-H1. Together these results describe a mechanism by which TGF- β may rapidly downregulate cdc25A activity to allow cell cycle arrest.

Discussion

The TGF- β s inhibit proliferation of a variety of normal cell types, including most epithelial cells and hematopoietic cells (Akhurst and Derynck, 2001). However, non-transformed dermal fibroblasts and fibroblastic NIH3T3 cells are growth stimulated by TGF- β (Clark et al., 1997; Sgambato et al., 1997). Here we report that restoration of RhoA signaling through p160^{ROCK} in NIH3T3 cells converts the growth response to TGF- β treatment from growth stimulation to growth inhibition. It is further demonstrated that inhibition of p160^{ROCK} in NMuMG cells blocks the G1 arrest induced by TGF- β . Investigations of the down stream effectors of RhoA/ p160^{ROCK}-mediated growth arrest demonstrated that TGF- β rapidly stimulated p160^{ROCK} translocation to the nucleus, phosphorylation, and inhibition of the cdk-activating phosphatase, cdc25A (Hoffmann et al., 1994; Jinno et al., 1994). This was associated with inhibition of cyclin E/cdk2 kinase activity, which likely plays a causal role in the inhibition of G1-S progression. These results provide evidence that signaling through RhoA and p160^{ROCK} is important in TGF- β inhibition of cell proliferation.

The NIH3T3 cells make an interesting model system to examine TGF- β signaling since they are responsive to Smad signaling, yet refractory to TGF- β -mediated growth inhibition and RhoA activation (Figure 1). Because we were specifically interested in TGF- β -mediated RhoA signaling which is associated with a transient increase in RhoA activation, a constitutively active RhoA (V14RhoA) was purposely not expressed. We also wanted to avoid epithelial differentiation of NIH3T3 cells, as has been reported to occur under V14RhoA expression (Sander et al., 1999). The introduction of wild type RhoA attenuated TGF- β -mediated growth stimulation. But, the expression of the Rho GEF, Ost, in NIH3T3 cells resulted in TGF- β -stimulated RhoA activity and the rescue of growth inhibitory properties of TGF- β (Fig 2). Although Ost is clearly not the endogenous GEF in these cells, the data suggest Ost is able to couple to the TGF- β signaling pathway. It further suggested that TGF- β regulation of cyclin E associated kinase activity is a result of the lack of TGF- β -mediated RhoA activation. We also found the coincident inhibition of TGF- β -stimulated RhoA activity and growth inhibition achieved by the expression of the rhotekin RhoA-binding domain (RBD) (Ren et al., 1999; Welsh et al., 2001) in NMuMG cells (Fig 2). These findings complement recent studies showing that blocking RhoA and p160^{ROCK} activity resulted in early cyclin D1 expression and accelerated G1-S progression (Welsh et al., 2001) and further support our hypothesis for the requirement of RhoA activity in TGF- β -mediated growth arrest. Further, RhoA signaling is an immediate mechanism of cdc25A enzymatic inhibition through post-translational modification that precedes the previously described TGF- β -mediated transcriptional down regulation of cdc25A (Iavarone and Massague, 1997).

Thus a two step model for TGF- β -mediated G1 checkpoint inhibition can be proposed where there is an initial mechanism for growth inhibition by way enzymatic inhibition of cdc25A followed by a secondary response associated with the various transcriptional up-regulation of cdk-inhibitory

proteins (Datto et al., 1995; Reynisdottir et al., 1995; Toyoshima and Hunter, 1994) and down-regulation of *c-myc* (Coffey et al., 1988) and *cdc25A* genes (Figure 7). The potential for TGF- β -mediated phosphorylation and inhibition of *cdc25A* phosphatase activity is illustrated by the *in vivo* and *in vitro* kinase assays (Figures 5) and *cdc25A* phosphatase assay (Figure 6B), respectively. As suggested by the cell fractionation results in Figure 6A, p160^{ROCK} translocates to the nucleus in order to inactivate *cdc25A*. P160^{ROCK} translocation is presumably mediated through its activation by RhoA and the presence of a bipartite nuclear localization signal sequence at position 1020aa-1037aa. An analogous "two-wave" concept of G1-S inhibition during genotoxic stress is also found to target *cdc25A* (Bartek and Lukas, 2001). Chk1 and Chk2 proteins are reported to inactivate *cdc25A* by serine phosphorylation as a result of DNA damage (Falck et al., 2001; O'Neill et al., 2002; Sanchez et al., 1997). This may suggest that the preservation of genomic integrity by growth arrest is a fundamental response to both TGF- β -mediated transdifferentiation and DNA damage.

The context dependence of RhoA-p160^{ROCK} activation in TGF- β -mediated EMT and growth arrest is particularly intriguing. We previously reported that RhoA and p160^{ROCK} are essential in TGF- β -mediated EMT of NMuMG cells (Bhowmick et al., 2001a) in accord with its well-established role in actin cytoskeletal organization (Hall, 1998). Here we also provide evidence for RhoA and p160^{ROCK} involvement in TGF- β -induced growth inhibition. However, Rho-family GTPases have been implicated previously in the positive regulation of cell cycle progression through the G1 phase, specifically, in Ras-mediated transformation as well as LPA stimulation of RhoA (Kranenborg and Moolenaar, 2001). The apparent discrepancy illustrates the complexity of the biological effects of RhoA and p160^{ROCK} activation.

Experimental Procedures

Cell Culture. The NMuMG and NIH3T3 cells were purchased from the American Type Culture Collection (Rockville, MD) and propagated in DMEM with 10% fetal bovine serum. Insulin (10 μ g/ml) was supplemented to the NMuMG media. The dominant-negative p160^{ROCK} cDNA (from Dr. Shuh Narumiya, Kyoto University) subcloned into pBabe, retroviral vector, was transfected into amphotrophic into retrovirus producing Phoenix cells (from Dr. Gary Nolan, Stanford U.). Conditioned media was allowed to incubate with target cells for 24 h after which point the media was replaced for subsequent experiments (Bhowmick et al., 2001a; Kinsella and Nolan, 1996). Experiments were performed 48 h after transfection or infection.

RhoA-GTP assay. Activation of RhoA was detected in NMuMG and NIH3T3 cells by adsorbing cell lysates to Rho binding domain of rhotekin (RBD) to enrich for GTP-bound RhoA as described previously (Bhowmick et al., 2001a; Ren et al., 1999). Adsorbed and non-adsorbed lysates were then western blotted for RhoA (26C4, Santa Cruz Biotechnology, Santa Cruz, CA).

Thymidine Incorporation, Flow Cytometry, and Sequential Cell Counting. As described previously, cells treated for 48 h with TGF- β were pulsed with 3 H-thymidine two hours prior to harvest. [3 H] incorporation was measured by scintillation counting to assess DNA synthesis (Bhowmick et al., 2001a). Cells treated for 24h with or without TGF- β were stained with propidium iodide in sodium citrate buffer (containing 0.1% Triton X-100 and 5 μ g/ml RNase A) for 30 min prior to flow cytometric. A total of 50,000 cells per sample were analyzed by flow cytometry on a FACS-Calibur (Becton Dickinson Biosciences, San Jose, CA) and cell cycle profiles determined using Cell Quest software.

Sequential cell counts were performed through a 5 day time course using a Coulter counter.